REVIEW ARTICLE

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Methods of analysis of gut microorganism – actual state of knowledge

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Abstract

Introduction. Microbiota plays an integral part in maintaining organism homeostasis, through eliminat pathogens, anti-cancer activity, synthesis of digestive enzymes and vitamins, maintaining the continuity of the intestinal epithelium and stimulation of the gastrointestinal immune system, and encourage a quicker and more efficient immune response. Changes in the microbiota composition is often observed in patients with allergy, atopy, irritable bowel syndrome and other diseases, which is the reason for a growing interest in methods of identification of the gut microbial complex.

Objective. The aim of the study was to compare the state of current knowledge about two methods used in the study of intestinal microorganisms complex: the traditional culture method and genetic analysis.

Description of the state of knowledge. Both techniques have advantages and disadvantages. The biggest limitation of the culture method is its inability to detect a significant number of the intestinal microbes. Using the microbiological technique we can only detect identifiable bacteria that can be grown on available substrates. For an accurate quantitative and qualitative investigation of the total microbiota, the more expensive genetic method is required. Due to genetic analysis it is possible to identify the vast number of new microorganisms and identify the dominant bacterial groups in different parts of the gastrointestinal tract.

Summary. Each of the presented techniques plays specific role in medicine and science. The combination of both methods may become a critical element for understanding the ecosystem of intestinal bacteria.

Key words

gut microorganisms, gastrointestinal tract, detection, PCR, culture

INTRODUCTION

Microbes that live in the gastrointestinal tract are an integral part in maintaining organism homeostasis. The host's own autochthonous microflora play several important roles. Well-adapted to the conditions in the gastrointestinal tract, gut microbes efficiently eliminate pathogens through interspecies competition. The ability to eliminate pathogens from the gastrointestinal tract is important not only for anti-infective reasons; some pathogens show carcinogenic activity (synthesis of enzymes involved in carcinogenesis – the production of metabolites toxic to humans). The anti-cancer activity of intestinal microorganisms is based on their ability to disable enzymes involved in carcinogenesis, the elimination of bacteria synthesizing harmful compounds and the degradation of existing carcinogens.

An important function of the intestinal ecosystem is metabolic activity – synthesis of digestive enzymes and vitamins; the production of compounds feeding the intestinal epithelium (e.g., short chain fatty acids, polyamides) or the ability to increase mucin synthesis contribute to maintaining the continuity of the intestinal epithelium. This, together with the overlying layer of mucus, creates an important line of defence, a significant barrier to prevent the penetration of microorganisms or allergens into the circulatory system.

One of the most important properties of intestinal bacteria is their impact on the gastrointestinal immune system (GALT, Gut Associated Lymphoid Tissue). With modulation exerted *in situ*, microorganisms encourage a quicker and more efficient immune response in response to the antigens. The impact of intestinal microbes on the human body is wide ranging, and their presence is essential for maintaining a state of good health. Disorders of the autochthonous flora are believed to be the cause of an increasing number of diseases. Impaired quantitative and qualitative composition of this ecosystem results in lower immunity, and an increased risk of fungal colonization and gastro–intestinal disorders. It is believed that an abnormal microbiota composition is the cause of inflammatory bowel diseases and may underlie the pathogenesis of autism. Pathology of the gastrointestinal microbial composition can be observed in patients with atopy, food and inhalation allergy, and in patients with irritable bowel syndrome. Hence, there is growing interest in methods of identification of the gut microbial complex as a whole, and analysis of the qualitative and quantitative dependence of bacteria present in the gastrointestinal tract. For a full understanding of gut microbiology it is also necessary to identify the interactions between individual elements of the ecosystem *in vivo*. We already know that this knowledge is essential for understanding the root of numerous diseases and may contribute to the development of innovative therapies.

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OBJECTIVE

The aim of the research was to compare two methods used in the study of the intestinal microorganisms complex: traditional culture method and genetic analysis.

STATE OF KNOWLEDGE

Distribution of microorganisms in the gastrointestinal tract. Intestinal microorganisms constitute a rich ecosystem, with about 17 families, 50 genera and more than 1,000 species of bacteria. Their composition, which depends largely on environmental conditions, is also variable between individuals. Significant effects on the gastrointestinal tract microbiota are methods of delivery, lifestyle, diet, medication, levels of stress, invasive medical procedures among others. Qualitative and quantitative microbiota dependence changes during the life of the individual. The strongest rearrangements can be observed during childhood and during aging. During delivery, newborns are colonized with micro-organisms from their mothers' reproductive tract and those occurring in the hospital environment [1]. Breastfed infants have a particularly high percentage of bacteria of the genus *Bifidobacterium* and *Bacteroides*, whereas feeding with modified milk compounds promotes the growth of *Escherichia coli* and *Clostridium*. A relatively mature system of micro-organisms is established at the age of about three years and at about seven years is typical for each individual. Other important changes take place during aging – a significant decrease in the number of bacteria of the *Bacteroides* and *Bifidobacterium* genus, and an increase in the number of *Clostridium*, *Eubacterium* and *Fusobacterium,* which are capable of producing potential carcinogens [2]. Although almost every segment of the digestive system is inhabited by microbes, the distribution of individual species is different. Depending on the conditions occurring in the various sections of the digestive system, the dominance of facultative aerobic bacteria or compulsory or facultative anaerobes can be observed. The meaningful fact is that nearly 99% of the total intestinal ecosystem is colonized by anaerobes. In the oral cavity and pharynx there is a large group of bacteria with different oxygen requirements. At the base of tongue and posterior pharyngeal wall coccidia *Rothia mucilaginosa* are the most numerous, and streptococci colonize dental plaque, especially *Streptococcus mutans* and *Streptococcus milleri*. Also the presence of stick like *Actinomyces* can be observed. Microbes in the oesophagus and stomach are transient, arriving with swallowed material. In a healthy person, the stomach should be the organ with low colonization ($<10^3$ organisms per millilitre of gastric contents), because the low pH is not conducive for the multiplication of most bacteria. Few microorganisms are in the majority among the optional anaerobes (*Staphylococcus spp, Streptococcus spp, Enterococcus spp* and *Lactobacillus spp*.). Strictly anaerobic microbes usually occur sporadically. The number of bacteria increases in the small intestine. Its initial segments – the duodenum, jejunum and ileum – in the context of colonization do not differ substantially from the stomach. This limited distribution of microorganisms is mainly due to strong peristaltic movements and the presence of bile and pancreatic juices. In turn, the further section, the small intestine, is a much more favourable microbiological niche. Its ecosystem is similar to that of the colon. The numbers of microorganisms is in the range 10^6 – 10^7 cfu/g of faeces and are mostly anaerobic organisms (*Clostridium spp, Bifidobacterium spp*, etc.). The large intestine has the optimal environment for growth of most bacteria resulting in their most frequent occurrence here (10^{11} – 10^{12} cfu/g faeces). The reason is the slowing of the vermicular movement and pH close to neutral. Colon microbiota is the richest and most diverse ecosystem of the whole organism. There are in particular facultative and strictly anaerobic bacteria. However, the dominant flora (about 30 – 40% of total) comprises only a few genera: *Bacteroides, Bifidobacterium, Clostridium*, and to a lesser extent, *Enterococcus, Escherichia coli* and *Lactobacillus*. Other large bowel microorganisms are variable between individuals, characteristic for the host [3].

Differences are also observed in the composition of microbiota related to the surface intestinal epithelium (MAM – Mucosa Associated Microbiota) and occurring in the intestinal lumen and mucus. It was shown that the composition of microbiota in mucus does not differ significantly from microorganisms in the intestinal lumen. Differences are observed between the bacteria in the mucus (where there is some availability of oxygen from tissues), and microorganisms associated with the epithelium. In the gastrointestinal tract also are present microorganisms associated with particles of food [4, 5]. Essentially, the composition of bacteria colonizing the large intestine to some extent corresponds to the flora present in the faeces. It should be noted that the comparison is not entirely adequate, especially due to the absence of bacteria related to the epithelium in the faeces.

Methods of detection of intestinal bacteria. The variability and diversity of bacteria colonizing the individual sections of the gastrointestinal tract is one of the main difficulties in the study of the intestinal ecosystem. A traditionally used method remains the diagnostic gold standard for many bacterial infections, and the method against which other tests are often evaluated. Specificity of culture is 100% in case of absent etiological factors in the prevalence of the healthy, but the sensitivity of culture is more difficult to determine and may be low [6].

Using this method allows one to obtain single colonies of certain species of bacteria on microbiological media. Some of the media already allow for the isolation, counting, and presumptive identification of bacteria grown at any one time. The addition of various substrates, nutrients, chromogenics and antibiotics allows for the growth of microorganisms with specific characteristics. For example the identification of *E. coli* can be made after several hours, based on the appearance of colonies obtained on media containing substrates for two specific enzymes – β-glucuronidase and β-galactosidase – and contained in the substrate chromogen, metabolized by these bacteria, giving a specific coloration to the colonies. In this way, the next stage of the biochemical identification of colonies can be skipped, which reduces the diagnostic time. The selective nature of microbiological chromogenic media allows also for faster detection in clinical microbiology of the so-called. ALERT – pathogens-bacteria, such as MRSA (methicillin-resistant *Staphylococcus aureus*), bacilli producing extended-spectrum enzyme-substrate (ESBL) or vancomycin – resistant coccidia of the genus *Enteroccous* (VRE) [7, 8]. Having a live microbial strain, its properties can be tested using different methods (biochemical,

immunological and genetic). Understanding the role of bacteria and their effects on host health still requires a microbiological culture. That is why new methods of culturing certain microbiota components, which thus far are difficult or impossible to keep, are sought. One of the solutions may be the satellite culture for demanding microorganisms around the bacteria producing necessary substances for their growth, as well as microculture techniques [9]. Culture methods allow us to differentiate live cells from dead cells. One of the methods to determine the number of live bacterial cells is serial dilutions of the test material in order to obtain a quantitative result, such as the number of bacterial cells per gram of faeces. It is believed that the stool preferably reflects conditions in the large intestine and is therefore material which represents the microbial ecosystem mentioned. Faecal material is easy and non-invasive to obtain. Microbiological analysis of the group of intestinal bacteria is also significantly cheaper than using molecular methods. However, a significant limitation of the method is its inability to visualize more than 90% of intestinal microorganisms. The reason is the lack of growth of intestinal bacteria on the available growth substrates, but also difficulties in providing an appropriate environment for the development of the culture, which are a suitable temperature, pH, osmotic pressure and oxygen content. In the latter case, the problem of providing adequate anaerobic conditions occurs at the stage of collection and transport, and further analysis of the material. Prolonged transport of stool samples before analysis may lead to significant changes in the composition of the bacteria present due to increased proliferation of certain types, especially those that tolerate oxygen. For this reason, the culture of faeces reveals considerable amounts of optional anaerobes. It has to be assumed that the number of obligatory anaerobic bacteria obtained from stool culture may be lower than actual because the stool collection is always associated with some exposure to air. We can assume that culturing is a method for assessing the portion of the microorganisms present in the faeces which we can grow. However, as previously mentioned, this analysis does not allow for the detection of microorganisms associated with the mucosal epithelium. It is assumed that microorganisms seen in the stool reflect the microbiology of the colon, particularly the descending colon and rectum. Microbiological culture does not allow us to learn about micro-organisms specific to the stomach, duodenum or jejunum. Conducted studies give conflicting results regarding the usefulness of culture in the evaluation of micro-organisms present in the colon. Cultures of samples taken from the intestine *post mortem* revealed the presence of significant differences in the number of bacteria between the distal part of the colon, and the caecum. Caecum pH is lower, which favours increased bacterial growth [10]. The study conducted by Marteau P. et al compared the results of the culture fluid of the caecum and faeces [11] with the fluid collected under anaerobic conditions and faecal samples collected with the preservation of the maximum possible anaerobic conditions. The study showed a significantly higher number of obligatory anaerobic bacteria – *Bacteroides* and *Bifidobacterium* in cultured stool than in the culture of intestinal fluid. Optionally, anaerobic bacteria accounted for only 1% of all anaerobes in the faeces and 25% in the fluid of the caecum. Significantly fewer species of bacteria of the genus *Lactobacillus* were observed in the faeces. Therefore, we cannot accurately portray the bacterial quantity and quality in individual parts of the gastrointestinal tract using this method of stool cultures. However, there is constant work to improve the standard methods of breeding and culture. In January of this year, a thesis about a new medium for obtaining intracellular pathogens in axenic (host cell-free) culture was published. In many cases, culture-dependent analyses should go hand-in-hand with culture-independent, genomics-based techniques [12]. However, as of today, the impossibility of a thorough analysis of the intestinal ecosystem by conventional culturing has led to the exploration of new research methods. There are high expectations regarding genetic analyses. A study by the National Institutes of Health GenBank in 2005, including an analysis of genes encoding 16SrRNA, allowed for the detection of 1822 bacterial sequences derived from the human gastrointestinal tract. Almost 93% of those were micro-organisms where identification with traditional culture methods was previously impossible [13]. One of the most commonly used molecular biology techniques is polymerase chain reaction (PCR) amplification of the coding sequence of 16S rRNA performed with a denaturing gradient gel electrophoresis (DGGE). In order to identify intestinal bacteria the use of the technique FISH – fluorescence *in situ* hybridization and DNA microarray methods can be applied. Polymerase chain reaction – PCR, invented by Kary Mullis et al. [14] in 1983, based on a sequence of repeated heating and cooling of the reaction – is one of the most useful methods of molecular biology. The qualitative analysis of PCR products is provided by DGGE electrophoresis using polyacrylamide gel, with an increasing concentration of DNA denaturant agent (urea). DNA molecules of different sequences migrate and then stop at a particular distance. Then, at a specific concentration of denaturing agent, DNA molecules change their structure (denaturation) which slows down the migration. Depending on differences in the sequence, variation in the speed of migration occurs at different positions in the gradient. Differences in the sequence will result in the appearance of characteristic band configurations in the gel – so-called fingerprints. Numerous variations of the basic PCR procedure have been developed, of which the most often used in microbiological diagnosis is Real-Time PCR (qPCR) and nested-PCR. The qPCR technique has very high sensitivity and specificity, and the ability to obtain a quantitative result [15]. High sensitivity and specificity are characteristic for the nested-PCR technique. It requires carrying out two PCR reactions and gel analysis of the product. The sequence repeated in the first reaction is used as a template in the second reaction (using primers located closer to the centre of the amplified DNA fragment). This significantly increases the specificity of the amplified product. Nested-PCR method was used in research by Kageyama [16] and analysis showed that the method described above (using the universal primer pair), allows for rapid identification of bacterial species, such as *Eubacterium rectale*, *Eubacterium eligens Eubacterium biforme* whose detection by traditional culture techniques is difficult. FISH method (fluorescence *in situ* hybridization) enables the detection of some genera and species using specific fluorescently labeled probes complementary to 16S rRNA sequence. An important advantage of this method is the possibility of rapid detection of even single cells of studied microorganisms in their natural environment. Microarray technology is also used in order to identify micro-organisms.

The principle of this procedure is relatively simple and based on the method of hybridization. Unlike the previously discussed techniques – FISH, DNA probes are placed on solid medium. Test samples derived from biological material are fluorescently labeled and applied to the probes. American researchers used the oligonucleotide microarray method for the detection of intestinal bacteria in faecal samples. Wang et al. [17] developed probes based on the 16S rRNA gene sequences for 20 species of intestinal bacteria, among which we can highlight: *E. coli, E. faecium, L. acidophilus, F. prausnitzii* et al. Researchers amplified the 16S rRNA gene sequence, using two universal primers. For detection they used three 40-nucleotide probes specific for each of the targeted species. The results of the survey clearly indicated that the method is an effective technique for detecting the dominant human intestinal bacteria in faecal samples, and can allow for the detection of dozens of species simultaneously.

Although molecular methods allow us to go one step further with respect to traditional culture methods, they are not without drawbacks. The basic element of the genetic analysis is the PCR technique. Also, the smaller the number of bacteria in a given material the weaker their determinability using the afore-mentioned technique. In turn, the fluorescence *in situ* hybridization does not always give sufficiently specific results. This happens in the case of the use of degenerate probes (ambiguous). The search for new species or strains of bacteria poses difficulties for determining the optimal hybridization conditions. Some bacterial cells present in the test environment will not be lysed during the reaction, and in consequence will not be included in the results of the study. The usefulness of the FISH technique to identify bacteria with a small number of ribosomes seems to be the only problem. In the case of the presence of ribosomes with a particularly compact structure, a high risk of incorrect hybridization occurs, which can consequently generate false-negative results [18,19]. False results can occur due to the multiplication of already dead fragments of strains during PCR analysis. Consequently, they are mistakenly included in the results of the study. Therefore, neither do genetic techniques constitute an excellent tool for the study of the complexity of microbiota. However, they are certainly an indispensable element for further progress. As already mentioned, the high cost of molecular techniques, far exceeding that of traditional methods of culturing, is a limitation. Currently, molecular analysis is a basic tool for understanding the complexity of the intestinal ecosystem, being qualitative and quantitative, and interactions between microorganisms in the mucus, in the gut lumen, and those associated with mucous membrane of the epithelium. Genetic techniques are the basis for a new science called metagenomics (genomics of microbial populations), where the objective is to understand the total population of microorganisms present in a particular environment. In this method, bacterial DNA (DNA of the bacteria), extracted from the natural habitat of microorganisms, is cloned in order to create genomic libraries. Thanks to metagenomics, the identification of a total pool bacteria in the gastrointestinal could be successful. This step is essential for further investigation of the role of individual microorganisms and practical use of this knowledge in the treatment process [20].

The latest development in molecular biology technologies, called Next-Generation Sequencing (NGS), allows the sequencing of whole microbial genomes or complex microbial communities with so-called barcodes in matter of days, with significantly less labour and lower cost per base than traditional sequencing. NGS gives sequence outputs in the range of hundreds of megabases to gigabases routinely, which can provide both qualitative and quantitative data [21]. This technology has a high impact on basic and clinical research and is now used in the Human Microbiome Project to analyze normal and diseased microbiomes [22]. Although still expensive, the ongoing developments in NGS driven by highly competitive technologies will soon make it available for routine clinical diagnostics.

SUMMARY

In the state of current knowledge there are two methods used in the study of intestinal microorganisms complex – the traditional culture method and genetic analysis. Both techniques have advantages and disadvantages. The biggest limitation of the microbiological technique is the inability to detect a significant number of intestinal microbes. With this method, we are only able to detect identifiable bacteria that can be grown on available substrates. Nevertheless, the stool culture is still a fundamental and widely used method of detection of intestinal microorganisms. While maintaining proper anaerobic conditions during collection, transport and culture, the culture technique is a tool for assessment of the microbial contents of the colon, which is a niche for the largest number of bacteria in the body. To analyze the occurrence and abundance of specific, already known micro-organisms, the breeding method fully meets this role. The method of microbiological culture is commonly used in the microbiological analysis of materials collected from hospital patients (Clinical Microbiology) or in the evaluation of indicator organisms in research studies [23,24]. However, for an accurate quantitative and qualitative investigation of the microbiology of the entire gastrointestinal tract, genetic testing is required. Due to genetic evaluation/examination it is possible not only to identify the vast number of new organisms, but also to identify the dominant bacterial groups in different parts of the gastrointestinal tract. The isolation of previously unknown strains will allow for the detection of pathogens that play an important role in the etiology of diseases. Molecular diagnostics can constitute to be an effective tool in the search for organisms beneficial to health, such as probiotic strains. Each of the presented techniques plays a specific role in medicine and science. A balanced and cautious combination of both techniques may become a critical element for understanding the ecosystem of intestinal bacteria.

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